Incorporation of bacteria in concrete: the case against MICP as a means for strength improvement

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Strength improvement of cement-based materials by the addition of bacteria has been reported over the past decade and has been mainly attributed to microbially induced calcite precipitation (MICP[1]). However, the ability of bacteria to survive, grow and retain their metabolic activity in concrete is questionable. This research aims to shed light on the mechanisms involved in the strength enhancement of cementitious materials that contain bacteria. The addition of different concentrations of live and dead cells of *Bacillus cohnii* in cement mortars led to an increase in flexural and compressive strength for the mortars containing both types of bacteria. Findings of the present study led to exclusion of MICP as the main cause of strength enhancement, disproving earlier theories. Other known hypotheses including the behaviour of bacteria as organic fibres or as nucleation sites are thoroughly discussed, and a new approach is proposed.

Keywords: Concrete, Bacteria, Compressive Strength, Calorimetry, Thermal Analysis

1 Introduction

[1] Microbially Induced Calcite Precipitation

Despite the initial negative perception of microorganisms' influence on building materials, the interaction between bacteria and materials such as concrete and stone has been reconsidered in the light of new potential applications. The basis of these applications lies in the ability of most bacteria to induce the precipitation of calcium carbonate, mainly in the form of calcite, through their metabolic activities under certain conditions [1]. Self-healing concrete and surface treatment of stone and concrete structures are major applications of microbially induced calcite precipitation (MICP) in the field of construction materials [2, 3]. To evaluate the suitability of using bacteria for crack repair applications in concrete, Ramachandran *et al.* [4] were the first to investigate the mechanical properties of bacterial concrete, that is concrete with bacteria incorporated in the matrix of the material. Since then, due to the promising results of these initial studies, more research has emerged focusing on the use of bacteria for strength and durability enhancement of concrete.

Inclusion of bacteria in cement-based materials has been investigated through different experimental methods, varying with the type of bacteria used, the presence of nutrients and the curing conditions applied. Alkaliphilic and alkalitolerant bacteria such as the ureolytic *Sporosarcina pasteurii* [5-7], *Bacillus megaterium* [8, 9] and *Bacillus sphaericus* [10] and the non-ureolytic *Bacillus cohnii* [11] and *Bacillus subtilis* [12, 13] are some of the -species that have been used. Bacteria are often introduced into the samples in a liquid medium containing the cells at the desired concentration along with nutrients and growth components like nitrogen, carbon and calcium sources [7]. This replaces the mixing water or part of it [10, 12] in mortar samples. Curing of bacterial mortar samples in a similar nutrient solution is also common practice in the above-mentioned studies, although water curing has also been applied [9, 13]. Despite differences in methods, the above-mentioned studies have recorded increases in strength varying from 17% [5] to 49% [11] after 28 days of curing in bacterial mortars when compared to mortars without bacteria. It has generally been assumed that MICP is responsible for this phenomenon. MICP was even considered as the driving mechanism for increased strength in studies where nutrients were not provided, and water was used instead of a nutrient liquid medium for introducing the cells into the mortar and for curing the samples [9, 13-16]. However, this interpretation is not consistent with the knowledge that MICP requires bacterial metabolism, which is unable to take place without nutrients. Therefore, this brings into question whether the past

interpretations of the reason for strength improvements have been correct. Indeed, Ramachandran *et al.* [4] found that the strength, particularly the 7-day strength, was increased even when dead bacteria cells, killed by autoclaving were added. This provides further evidence that strength improvements must be independent of MICP and that there must be alternative explanations for the strength improvements observed. On the other hand, there have been cases where the addition of bacteria had a neutral or even a negative effect on the strength of cementitious samples [17-19], either in the presence [18, 19] or the absence [17] of nutrients.

Bacteria's contribution to the process of calcite precipitation lies in their ability to influence the environmental conditions that surround them in a way that favours the precipitation of carbonates [20]. Metabolic activities of bacteria such as hydrolysis of urea and oxidation of organic sources, result in an increase of the pH and the dissolved inorganic carbon around the microorganisms [20], which in the presence of calcium leads to MICP. Their role as nucleation sites for mineral deposition has also been mentioned [21]. Negatively charged groups in the cell envelope of the bacteria in addition to the high surface-to-volume ratio of the cells lead to their high capacity of binding metal ions. In this case, calcite formation is not caused by bacterial metabolism and therefore dead cells could also be considered as capable of precipitation. In fact, it is conceivable that dead bacteria promote higher mineral deposition if cell lysis occurs, because more functional groups could be available for binding ions [22].

However, when it comes to concrete, the ability of bacteria to survive, grow, retain their metabolic activity and thus induce calcite precipitation in this environment is questionable. The high pH of concrete, the intense abrasion that takes place during the mixing process, stresses caused during setting and hardening, and the lower ingress of oxygen and nutrients as the material becomes less permeable all diminish the possibility of bacterial survival in the composite. This limitation has been acknowledged by various researchers [4, 23-26], and consequently the inclusion of the cells in protective materials prior to their incorporation in concrete was proposed early on by Bang *et al.* [23] and has been investigated since then.

Reasons other than MICP should, therefore, be considered for the increased strength noticed in bacterial concrete. Ramachandran *et al.* [4] ascribed the strength improvement that was recorded by the addition of dead bacteria to their behaviour as organic fibres, which, at an early age, would reinforce the material and increase its strength. On the other hand, no such positive effect was noticed by the addition of dead bacteria in other studies [7, 18]. Ghosh *et al.* [14] attributed the higher strength of the bacterial samples to the deposition of a fibrous filler material, which was identified as gehlenite in later studies [15]. Likewise, in work by Biswas *et al.* [27] the secretion of a bacterial protein with silicifying properties was considered responsible for the formation of additional silicate phases, like gehlenite, in the cementitious matrix. Elsewhere the role of bacteria as nucleation sites for the formation of additional hydration products and mineral phases was considered responsible for the improved microstructure and strength properties of the bacterial samples [28].

In this context, the present study examined the effect of live and dead bacterial cells of various concentrations on the mechanical properties of cement mortars, investigating in-depth the behaviour of bacteria in the composite. The findings lead to the exclusion of MICP as the driving force of strength improvement in bacterial concrete, contrarily to what is currently discussed in the literature. Thus, this paper shifts this conversation from MICP to alternative theories including the ability of bacteria to act as organic fibres, for bacteria to promote C-S-H formation via nucleation, and chemical effects related to the composition of Gram-positive bacteria. Overall, the research contributes to the understanding of the interaction between bacteria and cement-based materials, which is crucial for improving relevant technologies, such as bacteria-based self-healing concrete.

2 Materials and methods

2.1 Preparation of live and dead bacteria cells

Cells of the alkaliphilic and spore-forming bacterium *Bacillus cohnii* DSM 6307 were used in this study. As an aerobic bacterium, *B. cohnii* promotes calcite precipitation by oxidizing organic sources [3]. The cells were stored in 25% (v/v) glycerol at -80 °C. To routinely culture *B. cohnii*, lysogeny broth (LB) broth was mixed with 100 ml/l Na-sesquicarbonate (42 g/l $NaHCO_3$ and 53 g/l Na_2CO_3) to adjust

to pH 9.5. Bacterial cultures were grown at 30 °C, and liquid cultures were agitated at 150 rpm. Cells were grown overnight in 2 ml volumes and were used to inoculate (1:1000) larger volume flasks (200 ml). Growth of liquid cultures was monitored spectrophotometrically as optical density at 600 nm wavelength (OD_{600}) in cuvettes of 1 cm light path length. Cells were pooled to ensure that the starting cells for each experiment were the same and OD_{600} was determined. Cells were then divided into live and dead sets. Live cells were immediately collected. The cells for the dead set were killed by autoclaving at 121 °C for 15 minutes before collection. Cells were collected by centrifugation (3220 X g for 2 min at RT). Complete killing of cells by autoclaving was confirmed by viability testing using the plate count method. All preparations of bacteria were used within 24 hours of their production.

The number of viable cells per OD_{600} for *B. cohnii* grown under the described conditions was initially determined by plate counting of serial dilutions and resulted in a relationship of 8.88×10^8 cfu per ml per OD. This value was used throughout this study to calculate cell numbers from OD_{600} readings. The fraction of spores in a typical preparation of vegetative cells was 4.5×10^{-7}, as determined by carrying out viable cell counts of untreated cell preparations (total viable cells) and after 20 min of heat treatment at 80°C (spores), showing that our vegetative cell preparations were virtually free of spores.

2.2 Zeta potential measurement and cell wall integrity

Zeta potential measurements followed a protocol adapted from Soon *et al.* [29]. Volumes of 4 ml were taken from cells prepared in 2.1 and centrifuged at 4,000 ×g for 5 minutes. The supernatant was then discarded, and cell pellets were washed once in one volume of autoclaved distilled water. The cell pellet was re-suspended in autoclaved distilled water and a 2 ml volume was prepared to a final OD_{600} of 0.5. The zeta potential of 1 ml cell suspension was measured in DTS1061 cuvettes with a Zetasizer Nano ZS equipped with a 633 nm red laser (Malvern Instruments Ltd, Malvern, UK) using the Helmholtz-Smoluchowski theory. Measurements were performed at 25°C (120s calibration) and taken as triplicates (n=3) of three biological repeats.

To test for the integrity of the cell wall after autoclaving, live and dead cells were subjected to Gram-staining and imaging was carried out with a compound microscope at 1000-fold magnification.

2.3 Preparation of cement mortar specimens

Portland limestone cement, CEM II/A-L 32.5R (BS EN 197-1) and standard sand (BS EN 196-1), were used for preparing prismatic cement mortars specimens of 40x40x160 mm^3. Two series of bacterial samples were made, one with live bacteria cells, noted as BC, and one with dead bacteria cells, noted as DBC. Cells were subjected to serial dilutions with tap water until concentrations of 10^5, 10^7, and 10^9 cells/ml were achieved, both for live and for dead cells. Thus, the name of the samples refers to their content in live or dead bacteria and the concentration of the cells, e.g. $BC10^5$ for live cells with 10^5 cells/ml concentration of bacteria and $DBC10^5$ for dead bacteria cells with the same concentration. The water containing the cells was used as the mixing water for preparing bacterial samples at the three concentrations. Control samples, with no bacteria, were also prepared as a benchmark, and tap water was used for their preparation. In all cases, a water/cement ratio of 0.5 was used. Mixing was carried out in accordance with the BS EN 196-1, and the specimens were demoulded 24 hours after casting. A total of 63 samples were prepared, three for each mix design. After demoulding and until the day of testing all samples were cured in tap water at 20°C in separate containers to avoid any cross-contamination between the samples. Details on the materials used, the mix designs and the notation used throughout this paper are given in Table 1.

2.4 Viability of the bacteria in cement mortar

Cell viability was investigated for samples containing the largest concentration of live cells, i.e. 10^9 cells/ml, after mixing and before setting of the mortar. Three hours after the mixing, 1 g of wet mortar was resuspended in 1 ml of water and was then subjected to serial dilutions. An aliquot of 0.1 ml of the dilution was plated onto alkaline LB agar plates (pH 9.5, adjusted with 100 ml/l Na-sesquicarbonate as described in section 2.1) at 30°C and viable cells were subsequently counted.

2.5 Compressive and flexural strength

Compressive and flexural strength tests were carried out for all mortar specimens at 3, 7 and 28 days. Samples of each mix design were first tested in flexural strength in triplets and the split samples, 6 in total for each mix design, were subsequently tested for compressive strength. 50kN and 100kN hydraulic frames were used for testing flexural and compressive strength, respectively.

2.6 Hydration rate

The effect of live and dead bacteria on the hydration rate of the cement was studied for the three examined concentrations, 10^5, 10^7, and 10^9 cells/ml, with isothermal calorimeter analysis. Control and bacterial pastes were prepared using 30 g of cement and a water/cement ratio of 0.5. Tap water was used in the control sample, while bacterial samples were made with tap water with the addition of bacteria in three concentrations (10^5, 10^7, and 10^9 cells/ml). The test was carried out with I-Cal 4000 HPC isothermal conduction calorimeter. Cement pastes were placed in the calorimeter unit directly after hand mixing for a maximum of 60 seconds. The temperature in the unit remained 20˚C throughout the test, for a period of 72 hours.

2.7 Thermogravimetric analysis

Thermogravimetric analysis (TGA) was conducted for control pastes and for pastes containing live and dead bacteria. Based on the strength results, 10^7 cells/ml was chosen as an optimum bacterial concentration and was used for further tests both for live and dead cells. The pastes were prepared as described in section 2.6, using 30g of cement and a water/cement ratio of 0.5. After hand mixing the samples were cast in small plastic moulds of 125 x 100 mm^2 surface and 18 mm depth, where they were left to dry for 24 hours. Once demoulded, they were cured under water in separate containers at 20^{o}C for three days. For TGA measurements to be taken on 3-day-old samples, the hydration of the pastes was arrested on the third day of curing using the solvent exchange method described by Calabria-Holley *et al.* [30]. Specimens were removed from the water and were left to dry at room temperature for 15 minutes before being crushed to a size of 0.1-0.4 mm diameter. The pieces were then immersed in isopropyl alcohol, C_3H_7OH (isopropanol), of 99% purity for 24 hours.

Next, the samples were vacuum dried in a desiccator for a further 24 hours. Before being placed in the TGA, the pieces were further crushed with mortar and pestle into fine powder that could pass a 0.1 mm aperture sieve. Samples of 0.1 g, as weighed in a precision balance, were used for the analysis. TGA was conducted with Setsys Evolution TGA 16/18 (Setaram) and the samples were heated from 30˚C up to 1000˚C, at a rate of 10˚C/minute. To avoid any possibility of oxidation the analysis was carried out under the atmosphere of an inert gas (Ar) with a gas velocity 20 ml/minute.

Quantification of hydration products and calcite was performed using the TG-dTG curves that occurred from the analysis. The methodology proposed by Lothenbach *et al.* [31] was followed. Bound water was measured by taking the difference of the sample's mass before commencing the test - measured with a precision scale - and its mass at 500ºC as recorded during TGA. Portlandite ($Ca(OH)_2$) and calcite ($CaCO_3$) were calculated using their molecular masses as shown in Eq. 1 and 2 [31]. A relative error of ±7.5% due to the heterogeneity of the paste and the small amount of the samples used for the analysis (0.1 g) was considered.

$$Ca(OH)_2 = WL_{Ca(OH)_2} \times \frac{m_{Ca(OH)_2}}{m_{H_2O}} \quad (1)$$

$$CaCO_3 = WL_{CaCO_3} \times \frac{m_{CaCO_3}}{m_{CO_2}} \quad (2)$$

Where:

$WL_{Ca(OH)_2}$ and WL_{CaCO_3}: mass losses due to decomposition of $Ca(OH)_2$ and $CaCO_3$ respectively.

$m_{Ca(OH)_2}$, m_{H_2O}, m_{CaCO_3}, m_{CO_2}: molecular masses of the noted compounds, equal to 74, 18, 100, 44 g/mol respectively.

2.8 Microstructural analysis

Microstructural analysis was performed for control and bacterial cement pastes and mortars. Pastes that were prepared for TGA were subsequently used for microstructural analysis with a scanning electron microscopy (SEM). In addition, after compressive strength test at 3 and 7 days, fragments from the core of the mortars were taken for further analysis with SEM. Paste and mortar samples for SEM were prepared by breaking the fragments of the material into pieces of 0.1-0.4 mm diameter and arresting their hydration as described above. The pieces were then stored in small plastic bags in a

desiccator until the day of testing. For image acquisition the samples were coated with gold by sputtering for 3 minutes at room temperature. A JEOL JSM-6480LV scanning electron microscope was used and the accelerating voltage was set at 10 kV for imaging, while the emission current was 20 μA.

Table 1: Mix design (BS EN 196-1) and notation of mortar samples used in this study. BC and DBC stand for samples containing live and dead bacteria, respectively. Numbers in the name indicate the concentration of bacteria (cells/ml). Quantities are given for a triplet of samples.

Samples (symbol)	Cement (g)	Water (ml)	Sand (g)	Bacteria (cells/ml)
	CEM II/A-L 32.5R	Tap water	CEN Standard Sand	*B. Cohnii*
Control	450	225	1350	0
$BC10^5$	450	225	1350	10^5 -live cells
$BC10^7$	450	225	1350	10^7 -live cells
$BC10^9$	450	225	1350	10^9 -live cells
$DBC10^5$	450	225	1350	10^5 -dead cells
$DBC10^7$	450	225	1350	10^7 -dead cells
$DBC10^9$	450	225	1350	10^9 -dead cells

3 Results

3.1 Zeta potential and cell wall integrity

Zeta potential is an electrochemical property of bacteria commonly used to describe their surface charge. A bacterium's surface charge signifies its metal binding capacity by ions attracted to its surface. Figure 1 shows the results of zeta potential measurements for live and autoclaved cells. Both type of cells presented negative surface charge with the zeta potential being -55.79 mV and -45.53

mV for live and dead bacteria, respectively. The surface charge of autoclaved cells was less negative by 18%.

As a heat intensive process autoclaving leads to bacterial death by causing denaturation of their proteins. During this process changes to the surface chemistry of the bacteria are likely, which could explain the decrease of the surface charge noticed here. This degree of change is comparable to observations after severe heat treatment of another Gram-positive bacterium, *Staphylococcus aureus*, where it was shown that a 22% decrease in zeta potential was associated with significant changes in cell envelope permeability and thus likely structure [32]. To test if autoclaving had a wider impact on the integrity of the bacterial envelope, Gram-staining was carried out. As shown in Figure 2, both live and dead cells showed the same size and arrangement, the typical rod-shaped morphology of *B. cohnii* cells and the dark blue-purple staining (Gram positive) expected for intact cell walls. This showed that while autoclaving had killed the cells, it had not destroyed the integrity of the bacterial cell walls.

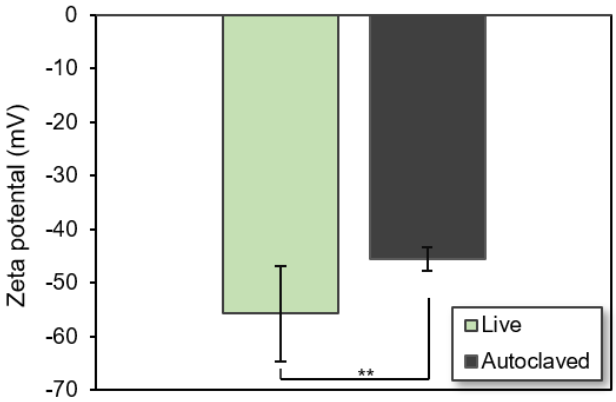
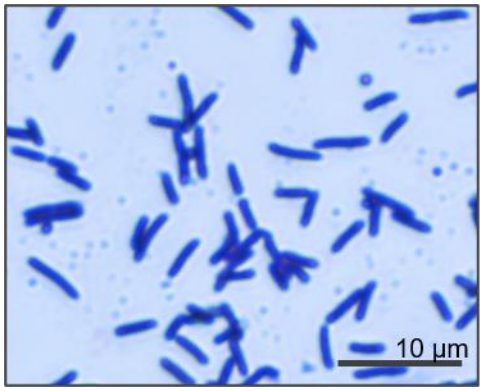
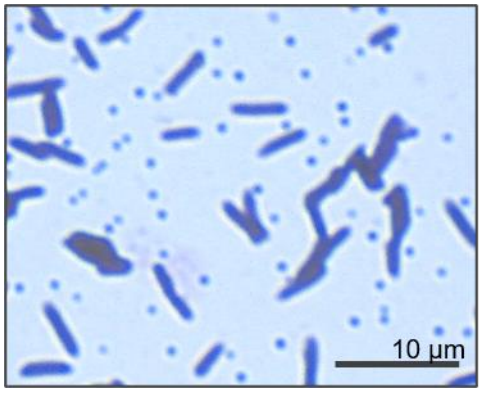


Figure 1: Zeta potential measurements of *B. cohnii*, live and autoclaved cells. An OD_{600} 0.5 live cell equivalent for each condition underwent measurements. The data represents the mean and standard deviation of three biological repeats each with three measurements. Statistics are representative of an un-paired t-test where ** is a $p \leq 0.01$.



(a)



(b)

Figure 2: Gram-stain of (a). live and (b). autoclaved *B. cohnii cells* (1000-fold magnification). The dark blue-purple colour of the rod-shaped bacteria is indicative of Gram-positive staining behaviour. The small blue specks are as common staining artefact from residual dye. A scale bar is indicated for size determination.

3.2 Viability of the bacteria in cement mortar

To estimate the number of bacteria that survived the mixing process when making cement mortar samples, the number of viable cells was counted shortly after the mixing. A total of 10^4 cells per gram of mortar were found to be viable 3 hours after the mixing took place. The examined mortar sample had originally contained live cells in a concentration of 10^9 cells/ml, which corresponds to a concentration of 1.11×10^8 cells per gram of mortar. Therefore, only 0.01% of the initial cell number were estimated to be viable directly after the mixing.

3.3 Compressive and flexural strength

Figure 3 shows the compressive strength results at 3, 7 and 28 days of cement mortar specimens containing live and autoclaved bacteria in different concentrations, and of control samples with no bacteria added. The percentage of strength change, Δ%, of the bacterial samples as compared to the control is also given. Similarly, flexural strength of the samples and the relative percentage of strength change are shown in Figure 4. At 3 days, mortars containing dead bacteria showed higher increase of compressive and flexural strength than the ones with live cells in comparison to the control, reaching a 35% and 24% increase in compressive and flexural strength respectively for 10^7 cells/ml bacterial concentration ($DBC10^7$). Samples with live cells at this age had a lower increase of strength or even decrease in the case of samples with 10^5 cells/ml bacterial concentration, for which compressive strength was decreased by 12%. On the other hand, at 7 days, samples with live cells – except for those with 10^5 cells/ml concentration of bacteria – performed better than the ones with dead bacteria, showing a 37% increase of compressive strength at 10^7 cells/ml concentration of bacteria ($BC10^7$). At 28 days, again, specimens with live bacteria of 10^7 cells/ml concentration ($BC10^7$) presented the highest strength improvement as compared to the control, by 32% and 22% for compressive and flexural strength, respectively. Mortars containing dead bacteria, also had higher strength than the control, especially the ones with bacterial concentration of 10^5 cells/ml ($DBC10^5$) for which compressive and flexural strength were increased by 19%. Overall, a significant enhancement of compressive and flexural strength of cement mortars is observed by the addition of bacteria cells, either live or dead. This is most pronounced for the dead bacteria in 3-day-old mortars and for the live bacteria at 7 and 28 days. There seems to be an optimum concentration both of live and dead cells at 10^7 cells/ml for which the strength increase was the highest. Finally, a slight decline in the strength improvement with time was noticed, mostly for mortars containing dead bacteria.

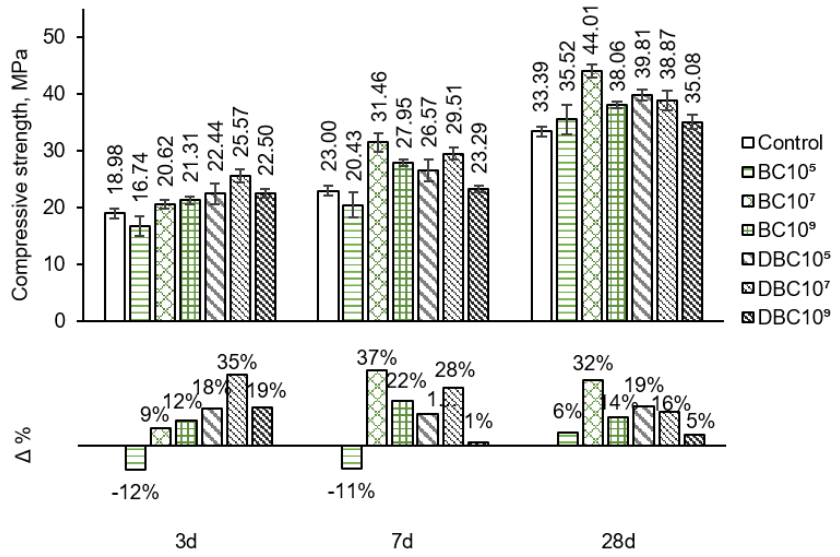


Figure 3: Compressive strength of control (no bacteria) and bacterial samples (BC series for live and DBC series for dead cells) in different concentrations (10^5, 10^7, 10^9 cells/ml) at 3, 7 and 28 days and percentage variation of the strength, Δ%, of the bacterial samples as compared to the control.

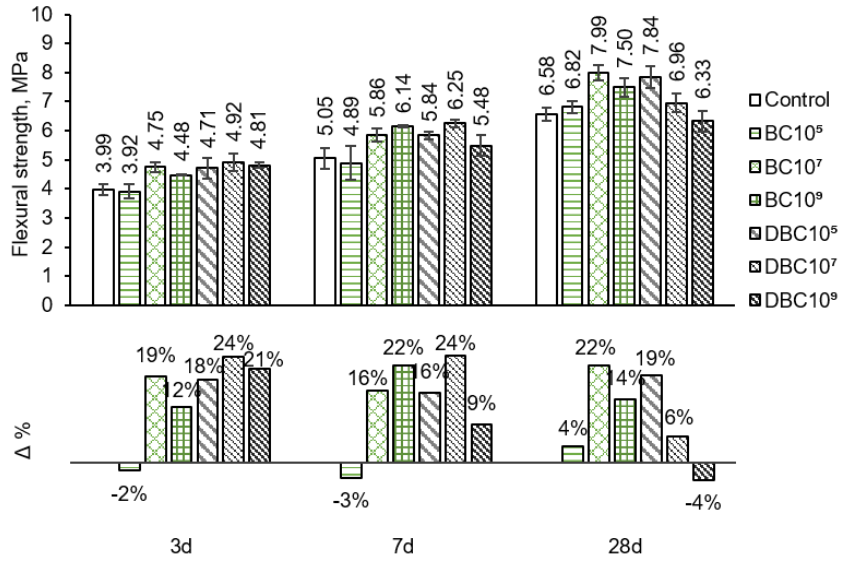
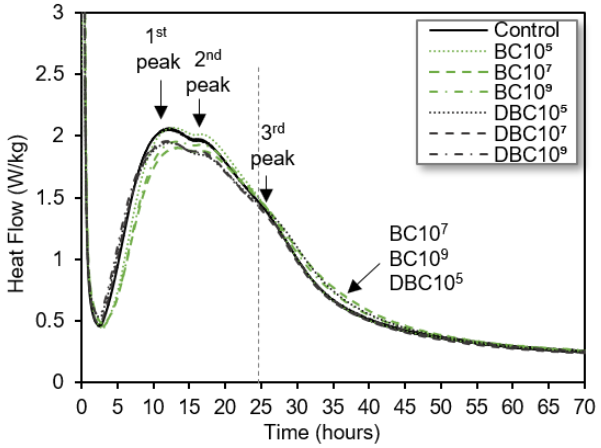


Figure 4: Flexural strength of the control (no bacteria) and bacterial samples (BC series for live and DBC series for dead cells) in different concentrations (10^5, 10^7, 10^9 cells/ml) at 3, 7 and 28 days and percentage variation of the strength, Δ%, of the bacterial samples as compared to the control.

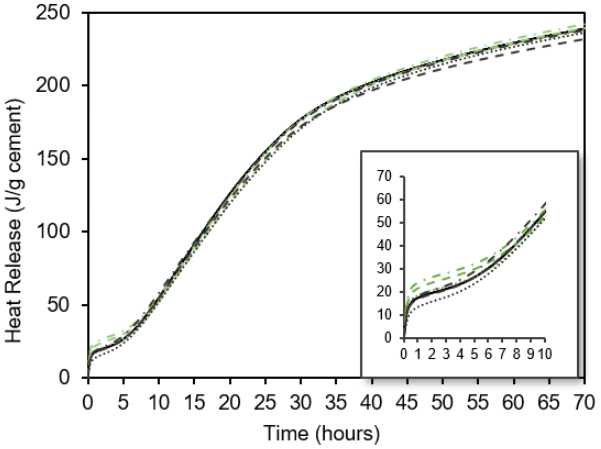
3.4 Hydration rate

Rate of heat evolution and cumulative heat release during the hydration of control and bacterial pastes are given in Figure 5a and 5b, respectively. After the rapid initial heat release in the first minutes of hydration, the heat flow slowed down during the induction period, between 1-3 hours, and then accelerated reaching the main heat evolution peak, shown as 1st peak in Figure 5a. During the acceleration period calcium silicate hydrate (C-S-H) and portlandite (CH) were formed at high rates due to the hydration of silicate phases of the clinker, mainly the alite (C_3S). Growth rate of C-S-H and CH became maximum at the 1st peak and then started to slow down. This peak occurred at approximately 12 hours of hydration for the control and the dead-bacteria laden samples. Samples with live cells, of 10^7 cells/ml concentration ($BC10^7$) in particular, showed a small delay in the 1st peak by 1 hour. The heat flow of the bacterial samples at this time appeared to be slightly lower than the control's, except for the $BC10^5$ sample which did not show any decrease.

After 25 hours of hydration certain bacterial samples, $BC10^7$ and $BC10^9$ with live and $DBC10^5$ with dead cells, presented a variation in comparison with the control paste, showing a shift in the hydration curve towards higher heat flows which extended up to 50 hours of hydration. For $DBC10^5$, a 3rd peak could be distinguished at 26 hours. Commonly, the appearance of the 3rd broad peak at around 25 hours of hydration is attributed to the formation of calcium monosulfoaluminate (an AFm phase) from the unreacted aluminate phase of the clinker (C_3A) and ettringite (AFt) [33]. These subtle changes were not depicted on the cumulative heat release results. As shown in Figure 5b, apart from a trivial increase in the heat release for samples with live cells noticed between 0-8 hours, cumulative heat release was not influenced by the presence of bacteria.



(a)



(b)

Figure 5: (a) Cumulative heat release and (b) rate of heat evolution, during the hydration of the control (no bacteria) and bacterial samples (BC series for live and DBC series for dead cells) in different concentrations (10^5, 10^7, 10^9 cells/ml).

3.5 Thermogravimetric analysis

Control and bacterial samples with live and dead cells of 10^7 cells/ml concentration were subjected to TGA at the age of three days. Results of the analysis are given in Figure 6, where mass loss over temperature curves (TG) and their derivatives (dTG) are shown for all samples. Three distinct troughs are noticed for all samples, indicated in the graph as I, II, III. The first, I, at 120^{o}C – 140^{o}C is related to

the loss of chemically bound water of cement hydrates, particularly C-S-H, ettringite and monosulfate [31]. II (440^{o}C – 480^{o}C) corresponds to the dihydroxylation of portlandite ($Ca(OH)_2$) and III (760^{o}C – 780^{o}C) to the decomposition of calcite ($CaCO_3$). In general, TG and dTG curves of control and bacterial samples appeared to be similar, with the three main troughs occurring at the same temperatures. However, a slight differentiation was noticed in the first trough for both bacterial samples implying a higher content of C-S-H in these mixes. Control samples presented an additional step in the decomposition of carbonates at around 675^{o}C, which did not occur in samples with bacteria. This extra weight loss step denotes the presence of mono- or hemicarbonates as well as the carbonation of portlandite in the control as opposed to the bacterial samples [31]. Bacterial pastes presented mass loss in temperatures higher than 810^{o}C, which according to the literature are related to decomposition of C-S-H to wollastonite ($CaSiO_3$) [34]. This further infers the increase of C-S-H content in the samples containing bacteria.

The results from the quantification of bound water (contained in C-S-H, AFt and AFm), portlandite and calcite are presented in Figure 7. An increase of the content of hydrates was noticed in bacterial samples when compared to the control, which was more pronounced for the sample with live bacteria. Bound water, related to calcium silicate hydrates, ettringite and monosulfate, was found to be slightly increased in bacterial pastes by 9.7% and 7.2% for live and dead bacteria bearing samples, respectively. Portlandite content was raised by 15.4% and 8.9% in samples containing live and dead bacteria, respectively. The increased content of hydrates (C-S-H, AFt, AFm and CH) could imply a higher degree of hydration for the bacterial samples at three days, particularly for samples with live bacteria at 10^7 cells/ml concentration. This was not confirmed, however, by the calorimetry analysis, as shown earlier. A small increase in calcite occurred for bacterial samples by 8.4% and 7.8% for samples with live and dead cells, respectively. It should be noted, however, that the second decarbonation trough observed for the control samples at ~650^{o}C was not taken into consideration.

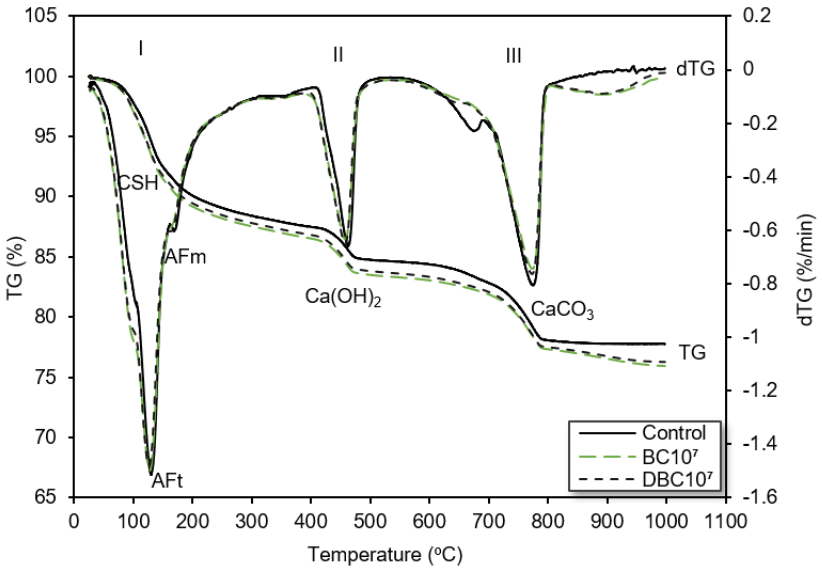


Figure 6: TGA/DTG of control paste (without bacteria) and pastes with live (BC) and dead bacteria (DBC) of 10^7 cells/ml concentration, at 3 days.

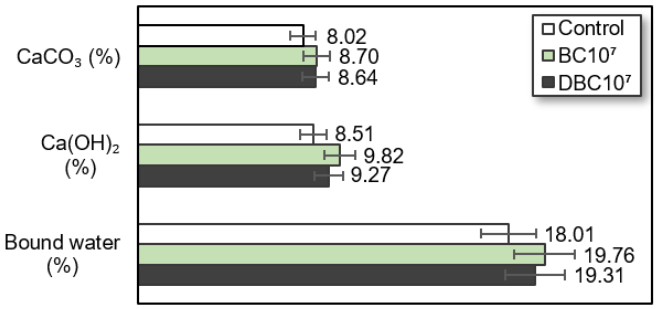


Figure 7: Quantification of the bound water (corresponding to C-S-H, AFt and AFm), portlandite ($Ca(OH)_2$) and calcite ($CaCO_3$) content of control and bacterial samples ($BC10^7$, $DBC10^7$) as occurred from the TG-dTG curves.

3.6 Microstructural and elemental analysis

Figure 8 presents SEM images of control and bacterial samples (10^7 cells/ml concentration) at 3 days and 7 days for pastes and mortars, respectively. Control paste samples showed areas of disruptions – highlighted with white arrows – which resulted in a relatively loose microstructure for the material. On the other hand, in bacterial samples, particularly with live cells, hydration products – needle-like AFt and foil-like C-S-H – were prominent, bridging the occurring voids and disruptions and thus creating a

coherent matrix. This agrees with TGA findings, where a higher degree of hydration was noticed for the bacterial samples. Mortar samples confirmed this observation. Hydrates, like CH platelets and AFt needles were obvious in control samples, however, voids and discontinuities were also present. Mortar with live cells presented a denser structure, with well dispersed hydration products. In the sample with dead cells, however, voids and absence of hydrates were noticed.

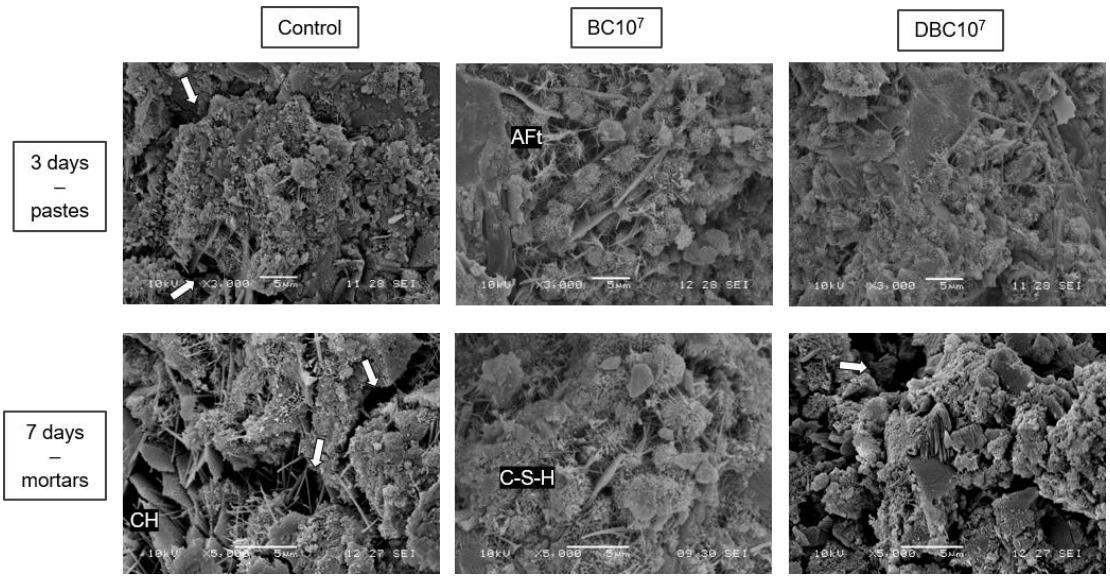


Figure 8: SEM images of control (without bacteria) and bacterial pastes and mortars with live (BC) and dead cells (DBC) of 10^7 cells/ml concentration. White arrows indicate areas of disruption.

4 Discussion

4.1 MICP

The present study showed that strength improvement in mortar samples can be achieved by using live or dead bacteria without nutrient addition. Therefore, the initial metabolic state of bacteria did not affect the strength outcome. In the absence of nutrients live cells are not expected to continue being metabolically active in the mortar, hence live and dead cells presented a similar behaviour. Pei *et al.*, on the other hand, observed that neither live nor dead bacteria had a positive effect on the strength of concrete specimens even though nutrients were provided in both cases [18]. Despite the contradictory

findings, which can be related to the different type of bacteria and experimental methods employed, the similar performance of live and dead cells remains. The opposite was noticed by Bundur *et al.* who found that, contrarily to live cells, dead bacteria did not lead to strength enhancement of cement-based mortars [7]. However, in this case dead bacteria were obtained by autoclaving the urea-yeast extract medium (UYE) medium that contained the cells and the autoclaved bacterial medium was then added in mortars replacing the water [7]. Thus, in addition to bacteria, other autoclaved components of the medium, like urea and yeast extract, could have influenced the strength results.

Viability of the bacteria is a key factor when examining MICP in cementitious materials. Here it was found that only 0.01% (10^4 cells/g) of the initial cell concentration survived the mixing process. Since a minimum concentration of 10^6 cells/ml has been shown to be essential for MICP to occur in cement pastes [35, 36], it is unlikely that it took place in the bacterial samples. Even though alkaliphilic bacteria were employed, the initial pH of cement mortar to which the cells were exposed is higher than their preferred levels of alkalinity. *B. cohnii* grows optimally at pH 10 [11], whilst the pH of fresh cement mortar is known to be approximately 13. Bundur *et al.* [26] examined the viability of *S. pasteurii* in mortar samples and reported that 0.1% of the initial concentration of bacterial cells remained viable after 7 days when the cells were suspended in tap water. The number of viable cells increased to 3% when a nutrient medium (UYE) was used for cell suspension instead of the mixing water. Achal *et al.* [8] observed that 0.05% of the initial number of *B. megaterium* cells suspended in nutrient medium survived in mortar samples after 28 days, but their number was higher in fly ash amended samples. Higher porosity of these samples favoured the aeration and consequently the survival of the bacteria in the material. It can be deduced, therefore, that high pH, in addition to lack of nutrients and oxygen led to the substantial loss of cells viability in this study. Although *B. cohnii* is a spore-forming bacterium, sporulation requires, among others, the presence of manganese [2, 37] and is unlikely to take place in the short time of the mixing. Even if spores did form, however, nutrients were not provided in the current study and would be necessary for germination and subsequent MICP.

Although MICP is primarily the result of bacterial metabolic activities, microbes can also contribute to calcite precipitation indirectly by serving as nucleation sites [21]. Cementitious materials are rich in

calcium cations, which can bind to the cells' surface and subsequently react with carbonate ions, also present in the material, to form calcite around the cells. However, in this study TGA results suggested that only a marginal increase of calcite content took place in samples containing live and dead bacteria, which was not confirmed by the microscopic analysis. This slight increase of calcite is insignificant considering the noticeable rise of strength in bacterial samples. Therefore, deposition of calcite, even of abiotic origin, is unlikely to be related to this strength enhancement.

Nevertheless, most studies in this field have reported evidence of calcite precipitation in samples with bacteria, with microscopic [6, 10, 38], and x-ray diffraction (XRD) [7, 10] analysis. However, Bundur *et al.* [7] reported calcite precipitation to be the result of the reaction between calcium ions in the cement with carbonates contained in the nutrient medium in which the cells were suspended. This would explain why calcite was noticed in the above-mentioned studies, where nutrient medium was used instead of water for introducing the bacteria in the mortar mix, while in studies where bacteria were suspended in the mixing water no additional calcite in bacterial samples was mentioned [14, 15].

4.2 Organic fibres

Apart from MICP, a physical effect of the bacterial cells on mortar samples was proposed by Ramachandran *et al.* [4] to explain the increased strength of the mortars with dead cells. In this theory, dead bacteria were regarded as organic fibres inside the cementitious matrix, which increase the early age strength of the material. In the long term, disintegration of these organic fibres leads to pores in the structure and thus to decreased strength. However, for assessing the physical contribution of the bacteria, their size and quantity in mortar samples should be considered. Table 2 shows the estimated total volume of the bacteria added in mortar samples prepared in this study. Volume size of a typical bacillus bacterial cell at 0.9 μm^3 [39] and average wet mass of a bacterial cell of $1x10^{-12}$ g [40] were considered for the calculations. The low volume of cells, reaching $2.6x10^{-5}$ % of the sample volume for the highest cell concentration, minimizes the possibility of any significant physical influence of the bacteria on the properties of the mortar.

Table 2: Estimated total cell volume and wet mass of the bacteria cells added in the mortar mixtures of one cement mortar sample (40x40x160 mm) in three different cell concentrations.

Cell concentration	Cells	Total cell volume	Total cell wet mass	Sample volume	Sample mass
(cells/ml)		(m^3)	(g)	(m^3)	(g)
10^5	$7.5x10^6$	$6.75x10^{-12}$	$7.5x10^{-6}$	0.256	675
10^7	$7.5x10^8$	$6.75x10^{-10}$	$7.5x10^{-4}$	0.256	675
10^9	$7.5x10^{10}$	$6.75x10^{-8}$	$7.5x10^{-2}$	0.256	675

4.3 Nucleation sites

Isothermal calorimetry revealed changes in bacterial cement related to the aluminate phases of the hydrated cement showing more monosulfate formed by the remaining C_3A and ettringite in these samples. On the other hand, TGA showed an early age increase in the hydration products, mainly of portlandite, of these pastes. The microstructure of bacterial mortars was found to be denser than the control's, particularly in samples where live cells were used. A similar observation was made by Chaurasia *et al.* [28] where additional formation of C-S-H and portlandite were reported in samples with bacteria. Elsewhere, bacterial addition was also found to affect the hydration products, promoting a more uniform distribution of the silicate phases in the matrix of the material and resulting in the formation of a new calcium aluminosilicate mineral identified as gehlenite [15].

The role of the bacteria as nucleation sites could potentially explain these results. Theoretically, their contribution would lie in promoting deposition of hydration products, rather than calcite as discussed in Section 4.2. A schematic depiction of this mechanism is shown in Figure 9a. Reactions between calcium cations bound in the cell wall with aluminum and ferrite anions could lead to the formation of more ettringite, some of which would be eventually converted to monosulfate, as observed in the calorimetry analysis. As more calcium reacts with hydroxide, silicate and aluminoferrite ions to form portlandite, C-S-H, ettringite and monosulfate, less calcium is available for mono- and hemicarbonates to form, thus they were not detected in bacterial pastes by TGA. Since surface

charge of live cells was found to be more negative than that of dead cells, the higher strength of samples with live bacteria can be explained by their increased binding capacity. However, the increase of hydration products recorded by calorimetry, TGA and SEM is relatively small for reflecting the significant strength improvement noticed in the respective samples. A denser C-S-H gel, even if its overall amount was only slightly increased, could explain this paradox [41]. In this context, further analysis for defining Ca/Si ratios of C-S-H as well as pore distribution in the examined samples could provide useful information.

4.4 Chemical interactions with cement

Another potential process through which bacteria may have contributed to strength enhancement of mortar samples is related to their composition. The cell wall of Gram-positive bacteria is primarily formed by a thick peptidoglycan layer, which is responsible for the stiffness of the wall, and teichoic acids [42]. Peptidoglycan consists of layers of carbohydrates linked with amino-acid chains, while teichoic acids are copolymers of glycerol phosphate and carbohydrates including residues such as alanine, succinate, pyruvate and choline acids [43]. Negatively charged phosphate groups of the teichoic acids are responsible for the attraction of metal ions to the bacterial cell wall. A schematic representation of the structure of the bacterial wall is given in Figure 9b. The environment of cement-based materials, including the high pH and the mechanical forces taking place during the mixing are likely to impose changes on the structure and the composition of the cell walls [44]. Alterations in bacterial gene transcription, in protein production as well as in the building blocks of the cell wall can be affected by the pH [44]. Unfolding, and consequently denaturation of bacterial proteins has also been reported at high (>10) pH levels [45]. Reaction between these bacterial constituents and the dissolved ions found in the cementitious solution may occur, affecting the properties of the material. Such reactions can occur even in the case of bacterial lysis, with the release of cell components like proteins and phospholipids in the cementitious matrix, as shown schematically in Figure 9a. Ghosh *et al.* [15] as well as Biswas *et al*.[27] found that a silica-leaching bacterial protein contributed to the formation of additional silicate hydrates in the cementitious matrix which fill the micropores increasing the strength of the material. Pei at al. [18] showed that cell walls of bacteria incorporated in cement mortar led to increased compressive strength. When peptidoglycan was added alone in mortar

samples, however, no effect was noticed on the strength performance of the material. The influence of other components of the bacterial cell wall on strength properties of mortars has not been investigated to date. This theory could be used to explain the results obtained in this study, in a similar way to that outlined in the previous section, 4.3. Both mechanisms could simultaneously occur in a complementary way as shown in Figure 9a.

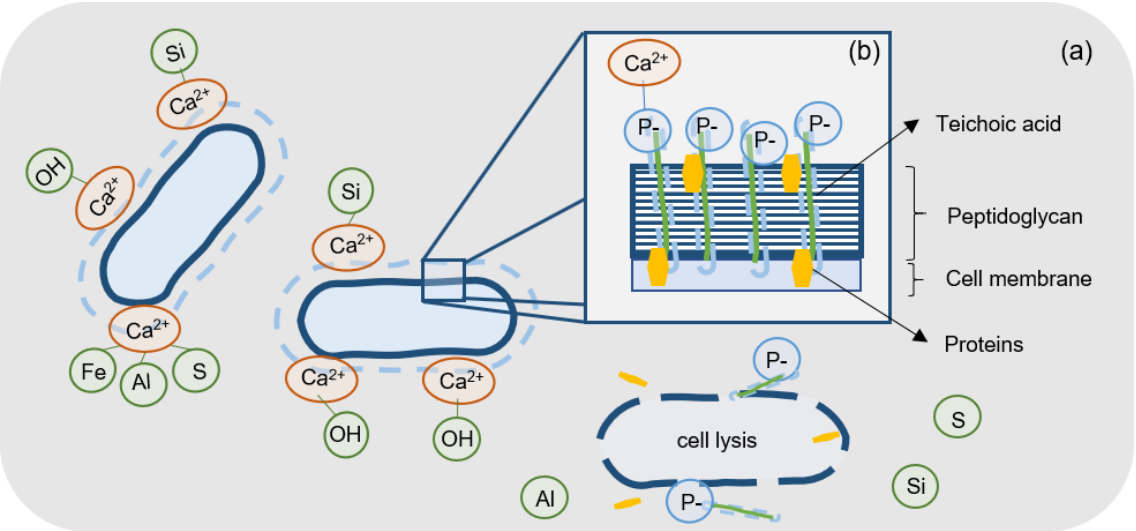


Figure 9: (a) Schematic representation of cementitious solution with cells of gram-positive bacteria (*B. cohnii* for this study). Binding function of the bacterial cell wall is shown with calcium cations (Ca^{2+}) adhered in the negatively charged cell wall. Dissolved anions (Si: $H_2SiO_4^{2-}$, Al: $Al(OH)_4^-$, S: SO_4^{2-} , OH^- , Fe: $Fe(OH)_4^-$) of the cement clinker react with bound Ca^{2+} to form hydrated products (ettringite, C-S-H, portlandite, monosulfate etc.). Bacteria act as nucleation sites propagating the formation of hydrates in the cementitious matrix. In the case of cell lysis components of the bacterial cell wall are released in the solution and may react with the present ions. (b) The structure of bacterial cell wall of gram-positive bacteria including its main components.

5 Conclusions

The present study investigated the influence of *B. cohnii* cells in cement-based materials regarding mechanical and physical properties of the latter. It was shown that bacterial cells lead to increased compressive and flexural strength in cement-based materials, even when nutrients required for their growth and survival are not provided.

Utilization of bacteria for strength improvement and for self-healing in cement-based materials should not be confused. MICP is the driving force in self-healing concrete and certain conditions need to be met for ensuring its occurrence, with viability of the bacteria and supply of nutrients being two important factors. On the other hand, it was shown here that bacterial viability is not a prerequisite for achieving higher strength in cementitious materials since strength enhancement is not the result of MICP. This is in direct contrast to arguments that have been proposed by much of the literature to date.

Instead, improvements in the mechanical performance of bacterial cement-based materials is presumably driven by chemical interactions between the cells and the dissolved ions of the cement clinker. This means that the outcome of such interactions highly depends on the type and the amount of the bacteria used as well as the composition of the cement. The water/cement ratio of the cementitious material as well as fineness of the cement and presence of additives and admixtures are factors that may influence the strength results of bacterial cement-based materials as they affect the availability of ions in the cement-bacteria system. Due to the complexity of this system, reactions with contradictory outcomes could occur, making the prediction of the final result harder. Further understanding of bacteria-cement interactions can enable promising applications of bacteria-based strength enhancement. A cheap and environmental-friendly solution for improving the mechanical properties of low-strength cementitious materials such as composites with low-carbon cements can be thus attained encouraging their wider use.

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